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# Transfer of auxinic herbicide resistance from wild mustard (*Sinapis arvensis*) into radish (*Raphanus sativus*) through embryo rescue

**J. Mithila and J. Christopher Hall[1]**
Kansas State University, Manhattan KS, USA, 66506
E-mail : mithila@k-state.edu

## ABSTRACT

**The discovery of auxinic herbicides (e.g., 2,4-D, Dicamba, Picloram) for selective control of broad-leaf weeds in cereal crops revolutionized modern agriculture. These herbicides are inexpensive and do not generally have prolonged residual activity in soil. Although cultivated species of Brassicaceae (e.g., radish and other vegetables) are susceptible to auxinic herbicides, some biotypes of wild mustard (*Sinapis arvensis*, 2n = 18) were found to be highly resistant to Picloram and Dicamba. Inter-generic hybrids between wild mustard and radish (*Raphanus sativus*, 2n = 18) were produced by traditional breeding coupled with *in vitro* embryo rescue/ovule culture. To increase frequency of embryo regeneration and hybrid plant production, several hundred reciprocal crosses were performed between these species. Upon altering cultural conditions and media composition, a high frequency of embryo regeneration and hybrid plant establishment was achieved. A protocol was also optimized for *in vitro* clonal multiplication of inter-generic hybrids produced by embryo rescue. To evaluate transfer of auxinic herbicide resistance from wild mustard into hybrid plants, several screening tests (involving *in vitro*, molecular-based as well as whole plant-based tests) were performed. Results indicated that hybrids of *R. sativus* x *S. arevensis* were resistant to auxinic herbicides suggesting, that, the resistance trait was transferred to these hybrids from the wild mustard. This research for the first time demonstrates the possibility of transfer of auxinic herbicide resistance from wild mustard to radish.**

**Key words:** Auxinic herbicide,embryo rescue, radish, resistance, transfer, wild mustard

## INTRODUCTION

Auxinic herbicides (e.g., 2,4-D, Dicamba and Picloram) were the first synthetic herbicides discovered and have been in use for more than seven decades. These have been a favorite choice among growers worldwide as they selectively control broad-leaf weeds in cereal crops. Owing to this, they are not recommended for use in commercially important dicot crops such as canola, radish or soybean. Biotypes of some broad-leaf weeds such as *kochia*, yellow Star Thistle, and wild mustard, have evolved resistance to auxinic herbicides due to selection pressure. Auxinic herbicide-resistant (R) wild mustard biotypes were found to be highly resistant to Picloram and Dicamba (104-fold) (Heap and Morrison, 1992). Genetic analysis of wild mustard auxinic herbicide resistance suggests that the resistance is determined by a single dominant gene (Jugulam *et al*, 2005; Jasienuik *et al*, 1995). Further, we recently reported identification of morphological and molecular markers linked to auxinic herbicide resistance in wild mustard (Mithila *et al*, 2012).

The family Brassicaceae consists of a number of economically important species, including oilseed canola, and vegetable crops such as cabbage, cauliflower, radish and broccoli. This family also comprises an excellent reservoir of genes for many economically important traits and is extremely conducive to gene transfer techniques. Radish is an important vegetable crop grown across the globe. Transfer of agronomically desirable traits among *Brassica* members, for example, between wild mustard and canola, has been previously reported (Bing *et al*, 1995; Inomata, 1988; Momotaz *et al*, 1998). However, to our knowledge, there are no reports describing transfer of agronomically important traits from wild mustard to radish. In this research, we tested the feasibility of transfer of auxinic herbicide resistance from wild mustard to radish following both traditional breeding and embryo rescue methods.

[1]Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

## MATERIAL AND METHODS

### Raising wild mustard and radish parental plants to produce hybrids

Auxinic herbicide -R wild mustard and -susceptible (S) radish plants were raised from seed. Seeds were sown in 6" plastic pots containing 'Promix', and placed in a growth chamber with 16-h photoperiod and 22/15°C day/night temperature. Light intensity and relative humidity were maintained at 350μmol·s$^{-1}$m$^{-2}$ and 65-75%, respectively. Each pot contained one plant and the plants were irrigated when required and fertilized weekly with 20:20:20 (N:P:K). To confirm resistance to Dicamba, wild mustard plants were treated with Dicamba (Banvel, BASF, USA) at 200g ae/ ha at three- to four-leaf stage of development (procedure for Dicamba application is described later; we used Dicamba in all experiments in this research, because it is a widely used auxinic herbicide in agriculture for control of broad-leaf weeds). All the plants survived Dicamba treatment and were used for further experimentation.

To produce hybrid plants between auxinic herbicide – R wild mustard and radish, reciprocal crosses were performed between these plants following the procedures described by Jugulam *et al* (2005) for wild mustard. Intergeneric hybrids failed to develop *in vivo*, as, the siliques (narrow, elongated seed-capsule) did not grow completely, i.e., it ceased growth before reaching maturity. Therefore, an *in vitro* method was sought for completion of embryo maturation and plantlet formation. To achieve plantlet regeneration via embryo rescue, we followed two strategies. In the first approach, siliques from crosses between Dicamba-R wild mustard and –S radish were harvested after ~10-12 days of pollination. These siliques were disinfested with 70% ethanol for 1–2 min, followed by 20% commercial bleach (sodium hypochlorite, 5.25%) containing three to four drops of Tween-20 for 15–20 min, and subsequently, rinsed four to five times with sterile deionized water. Embryos/ ovules from the siliques were excised aseptically with forceps and scalpel for culture on Petri dish containing 15 ml of one of the following two media: (A) MS (Murashige and Skoog, 1962) salts with Gamborg vitamins, sucrose (3%), 500 mg/l casein hydrolysate, or, (B) MS salts with Gamborg vitamins, sucrose (3%), 0.5 mg/l NAA, 2.5 mg/l Kinetin; pH of the media was adjusted to 5.8, and 8g of agar was added prior to autoclaving at 121°C for 20 min. In the second strategy, immature siliques were harvested 3-5 days post-pollination and surface-sterilized (as described above), followed by aseptic culture of the entire silique on medium A or B. The siliques were allowed to grow on the media for ~2 weeks, after which ovules were excised out from the siliques and cultured in a Petri dish containing fresh medium (A or B) for ovule maturation and regeneration into plantlets. All cultures were incubated in a growth room at 24°C under light (16-h photoperiod; 50μmol s$^{-1}$ m$^{-2}$) provided by cool, white fluorescent lamps (Philips Canada, Scarborough, ON).

After four weeks of embryo/ovule culture, regenerated hybrid plantlets were transferred individually to 'Magenta' boxes (300 ml plastic vessels, Magenta Corp., Chicago IL, USA) containing medium (C): MS salts with Gamborg vitamins and sucrose (1.5%); pH of medium C was also adjusted to 5.8, and 8g of agar was added before autoclaving at 121°C for 20 min. At four weeks upon transfer to medium C, the hybrid plants produced well-developed root and shoot system. At this stage, these hybrid plants were clonally multiplied by sub-culturing nodal segments in Magenta boxes containing medium C. *In vitro* hybrid plants with well-developed roots and shoots were transferred to soil ('Promix') and grown in a growth room under the same conditions as described, earlier for wild mustard and radish parental plants.

### Assessment of transfer of auxinic herbicide resistance into hybrids

***(i) In vitro assay:*** Previously, Mithila and Hall (2005) developed an *in vitro* assay to assess sensitivity of plants (explants) to auxinic herbicides. We used this protocol for initial screening to identify hybrid plants possessing auxinic herbicide resistance. For this assay, about 50-60 seeds of wild mustard auxinic herbicide-R and –S radish were surface-sterilized with 80% alcohol for 2-3 minutes and, then, treated with 30% bleach containing a drop of Tween-20, for 12-15 min. Subsequently, the seeds were rinsed 4-5 times with sterile distilled water. Sterilized seeds were cultured aseptically on medium C (described above) in Magenta boxes. Cultures were incubated in a growth room (under the same conditions as described above). After 6-7 weeks, stem segments of about 1 cm were excised aseptically using a scalpel from the seedlings of auxinic herbicide-R wild mustard and –S radish, as well as from clonally propagated hybrid plants, and cultured on medium (D) containing MS salts with Gamborg vitamins, sucrose (3%) and various doses of Dicamba or Picloram (0, 1, 5 or 10M); pH of medium D was adjusted to 5.8, and 8g of agar was added before autoclaving at 121°C for 20 min. At 3-4 weeks from initial culture, response of stem segments to Dicamba was recorded.

***(ii) Molecular assay:*** Recently, we developed a genetic map describing AFLP (amplified fragment length polymorphism) markers closely linked to auxinic herbicide resistance in wild mustard (Mithila *et al*, 2012). The two closest flanking regions on either side of R locus were located at a distance of 1.58 and –6.35 map units. These DNA markers were sequenced. To further assess transfer of auxinic herbicide resistance from wild mustard to radish, primers from the sequence of the closest marker (1.58 map units) were designed in the present stud (Forward primer: *GGCCGCGAGACATTGGTGA* and reverse primer: *TCTCTCGTGACCCTTTACAATTAG*) and synthesized. Genomic DNA was extracted from leaf tissue of hybrid plants as well as auxinic herbicide-R, -S wild mustard (auxinic herbicide-S wild mustard DNA was used as a negative control) and –S radish using DNeasy® Plant Mini (QIAGEN, Mississauga, ON, Canada) following manufacturer's protocol. Using the above-described primers, a PCR (polymerase chain reaction)-based assay was performed for detecting presence or absence of the PCR product (~220 bp length) that corresponded to the closest AFLP marker. The following PCR conditions were used: initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 45 seconds, with a final extension at 72°C for 7 min.

***(iii) Whole-plant based assay:*** Whole-plant screening was performed to validate transfer of auxinic herbicide resistance from wild mustard into hybrid plants. *In vitro* grown hybrid plants were established in soil. Auxinic herbicide-R wild mustard and –S radish plants were raised in a growth chamber as described earlier. Seedlings were treated with Dicamba (200g ae $ha^{-1}$) at three-to-four leaf stage of development using a motorized hood-sprayer. The sprayer was equipped with a flat-fan nozzle (8002 E) and calibrated to deliver 200 l/ha at 276 kPa. One and two weeks after treatment, the seedlings were visually rated for injury. Hybrid plants were classified as R or S by comparing the injury response with that in wild mustard auxinic herbicide-R and –S radish seedling response. Susceptibility of the plants to Dicamba was assessed based on symptoms of epinasty (downward curling of leaf and stem tissue) followed by death, whereas, R plants should show little or no response to Dicamba.

## RESULTS AND DISCUSSION

Inter-generic hybrids of radish and wild mustard failed to develop *in vivo* as the siliques were not able to grow completely, and ceased to develop ahead of maturity. Among the two strategies followed to produce inter-generic hybrids *in vitro*, the second strategy yielded more number of regenerated embryos than in the first approach (Table 1). There was no significant difference in the number of hybrids produced when embryos were cultured on medium A or B (Table 1). These inter-generic hybrids exhibited several morphological traits of both parents (*e.g.*, leaf shape, stem, plant height and flower colour, Fig.1). Clones of the hybrids were also successfully established *in vitro* by nodal cuttings. To our knowledge, this is the first report describing production of hybrids between wild mustard and radish via embryo rescue.

Sensitivity to auxinic herbicides results in excessive root growth in plant cultures *in vitro* (Mithila and Hall, 2005). In this study, after 3-4 weeks of initial culture of stem segments, excessive root formation was observed in Dicmaba-S radish in response to 1, 5 or 10µM Dicamba or Picloram in the medium. Conversely, wild mustard R segments did not show root formation even at 10µM Dicamba or Picloram (Fig. 2). More importantly, stem segments for all hybrids produced via embryo rescue responded similarly to R wild mustard when treated with Dicamba or Picloram (Fig. 2). These results suggest that

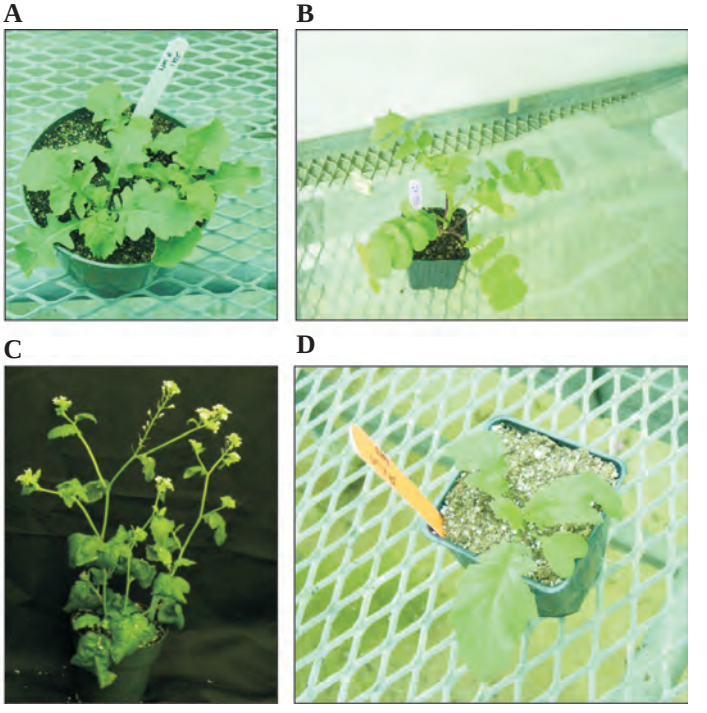


**Fig 1. Hybrids produced by crossing auxinic herbicide-S *R.-sativus* and -R *S. arvensis:* A and B represent *R. sativus* and *S. arvensis* plants, respectively. C and D are the inter-generic hybrids produced by crossing *R. sativus* and *S. arvensis* and following embryo rescue technique (note the hybrid exhibiting characteristics intermediate between *R. sativus* and *S. arvensis*)**

**Table 1. Hybrids produced by crossing auxinic herbicide-S *R. sativus* and -R *S. arvensis*: Frequency of embryo regeneration and hybrid plant establishment upon direct and reciprocal crossing followed by *in vitro* culture and embryo rescue**

| Cross combination | No. of buds pollinated | No. of siliques cultured | No. of embryos excised | No. of embryos germinated | No. of hybrids obtained |
|---|---|---|---|---|---|
| **Strategy I** | | | | | |
| *1. R. sativus* x *S. arvensis* | 100-150 | - | ~70 (A or B) | 2 | 2 |
| *2. S. arvensis* x *R. sativus* | 100-150 | - | ~65 (A or B) | 1 | 1 |
| **Strategy II** | | | | | |
| *1. R. sativus* x *S. arvensis* | 150-200 | ~ 125 | ~50 (A or B) | 5 | 5 |
| *2. S. arvensis* x *R. sativus* | 150-200 | ~ 110 | ~25 (A or B) | 1 | 1 |

A: MS salts + Gamborg vitamins (4.4. g/l) + 30 g sucrose/l + 500 mg/l casein hydrolysate (pH of the medium 5.8; agar 8 g/l)
B: MS salts + Gamborg vitmains (4.4 g/l) + 30g sucrose/l + 0.5mg/l NAA + 2.5mg/l kinetin (pH of the medium 5.8; agar 8 g/l)

all the hybrids derived via embryo rescue from crosses (both direct and reciprocal) between radish (S) x wild mustard (R) were found not sensitive to Dicamba or Picloram. Therefore, it appears that the R trait was transferred to the hybrids from R biotypes of the wild mustard. In addition, PCR results of molecular-based assay also demonstrated presence of a 220 bp fragment (representing the AFLP marker closely-linked to auxinic herbicide resistance in wild mustard; Mithila *et al*, 2012) in all the hybrids, and in Dicamba-R wild mustard; whereas, DNA of auxinic herbicide-S wild mustard and radish did not show this fragment (Fig. 3). These results suggest that the DNA fragment containing auxinic herbicide-resistance from wild mustard was probably transferred to R hybrids. Results from whole-plant screening further confirmed Dicamba resistance in the hybrids (Fig. 4) since these displayed little or no epinasty, a response that was similar to that in R wild mustard plants; however, radish plants ceased to grow 2 weeks after treatment with Dicamba (Fig. 4).

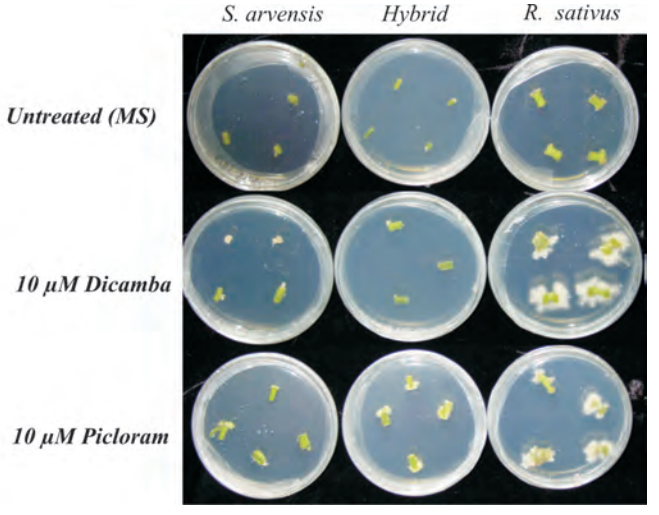


**Fig 2. Response to Dicamba (10µM) or Picloram (10µM) of stem segments of auxinic herbicide-R *S. arvensis*, -S radish (*R.-sativus*) and their hybrids (*R. sativus X S. arvensis*)**

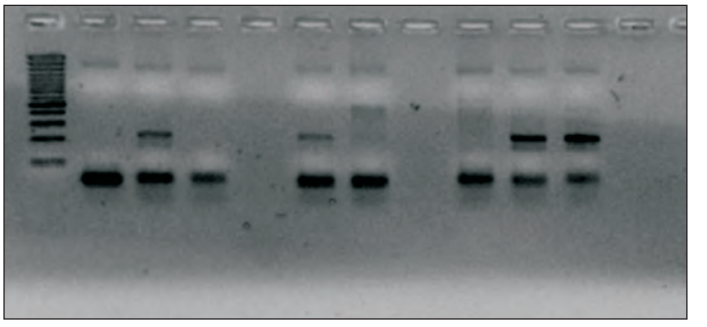

**Fig 3. PCR product (~220 bp) amplified by primers corresponding to a closet AFLP marker linked to Dicamba resistance in wild mustard (Mithila *et al*, 2012): Lane 1: 100 bp ladder; 2: reaction without a template; 3, 5: wild mustard auxinic herbicide-R; 4, 6: wild mustard-S biotype; 7: radish; 8, 9: inter-generic auxinic herbicide-R hybrids**

**Fig 4. A-C: Plants treated with Dicamba (200g ae/ha) 7 days after treatment. A and C illustrate dicamba- S radish and -R wild mustard, respectively; B represents the hybrid plant between radish (S) x wild mustard (R)**

Successful gene transfer from interspecific crosses using conventional plant breeding techniques involves several barriers, including (but not limited) to pollen-stigma incompatibility or sterility, embryo and endosperm imbalance, homologous chromosome pairing between the species, etc. In this research, we have demonstrated production of hydrids between two diploid species of Brassicaceae, viz., *S. arvensis* and *R. sativus*. Although both these species are diploid with 2n:18, genomes of these two species are different (Mizusima,1980). Success of interspecific hybridization among members of Brassicaceae depends on genome compatibility as well as ploidy of the species

(Mizusima, 1950). Based on cytogenetic analyses by Mizusima (1980), it appears that at least one chromosome of *S. arvensis* can form a bivalent with *R. sativus* during meiosis. Since all the hybrids produced via embry rescue in this research were found R to auxinic herbicides, the Dicamba-R gene residing on *S. arvensis* chromosome may have formed a bivalent with *R. sativus*, thereby transferring Dicamba resistance into radish.

In conclusion, in we have shown for the first time, possibility of production of Dicamba-R hybrids between *R. sativus* and *S. arvensis*. Further experiments are required to test the possibility of introgressing auxinic herbicide resistance from hybrids into radish by repeated backcross-breeding to develop radish varieties with desirable agronomic traits, along with auxinic herbicide resistance. This will allow selective removal of most broad-leaf weeds. Further, development of auxinic herbicide resistant radish following transfer of resistance trait from related species by introgreesion breeding is a non-transgenic approach thus, allowing acceptance across the globe, including Europe.

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